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Molecular characterization and immunomodulatory activity of sulfated fucans from *Agarum cribrosum*

MyoungLae Cho[a], Dong-Jin Lee[b], Jin-Kyung Kim[c], SangGuan You[b,*]

[a] East Sea Research Institute, Korea Institute of Ocean Science and Technology, Uljin 767-813, Gyeongbuk, Republic of Korea
[b] Department of Marine Food Science and Technology, Gangneung-Wonju National University, Gangneung 210-702, Gangwon, Republic of Korea
[c] Department of Biomedical Science, Catholic University of Daegu, Gyeongsan 712-702, Gyeongbuk, Republic of Korea

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ABSTRACT

The sulfated-fucans, known as fucoidans, were isolated from *Agarum cribrosum* and fractionated using ion-exchange chromatography to determine their molecular characteristics and *in vitro* immunomodulatory activity. The crude and fractionated fucoidans (F_1 and F_2) consisted mostly of carbohydrates (52.4–56.0%), sulfates (12.7–23.0%) and uronic acid (14.1–21.8%), with a small amount of proteins (3.9–9.3%), and included various levels of fucose (44.0–46.7%), mannose (18.9–26.8%), galactose (16.8–33.0%), xylose (10.7–17.0%) and glucose (3.5–9.5%). The crude and fractionated fucans contained one or two subfractions with average molecular weights (M_w) ranging from 110.1×10^3 to 2420×10^3 g/mol. The fractionated fucoidan, especially the F_1 fraction, strongly stimulated murine macrophages (Raw 264.7 cells), producing a considerable amount of nitric oxide (NO) and inducing expression of inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2) and interleukin-10 (IL-10) transcripts by activation of nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs) pathways. The maximally immunoenhancing F_1 fraction was mainly composed of (1 → 3)-linked fucose, (1 → 2)-linked mannose and (1 → 4)-linked glucuronic acid with sulfates at C-2 or both the C-2 and C-4 positions in (1 → 2,3)- and (1 → 2,3,4)-linked fucose residues.

1. Introduction

The sulfated-fucans, called fucoidans, are complex, heterogeneous and bioactive macromolecules consisting mainly of α-L-fucopyranosyl residues with a covalently linked sulfate, and are commonly found in the cell walls of brown algae (Mourao, 2007). The structural backbones of fucoidans have been largely classified into two groups, including (1 → 3)-linked α-L-fucopyranose residues from *Laminaria saccharina*, *Cladosiphon okamuranus* and *Chorda filum*, and (1 → 3)- and (1 → 4)-linked α-L-fucopyranose residues from *Ascophyllum nodosum* and *Fucus* spp. (Jiao, Yu, Zhang, & Ewart, 2011). However, species-specific fucoidans have been found with variation in their glycosidic patterns, monosaccharide units, sulfate content and site of sulfation (Costa et al., 2010; Pereira, Vilela-Silva, Valente, & Mourao, 2002). In addition, differences in uronic acid content and in branching levels impose a further complexity in the study of fucoidans (Bilan & Usov, 2008).

Fucoidans have garnered attention mainly because of their diverse biological activities, including antioxidant, anticoagulant, antiviral, anticancer and immunomodulatory activities (Senthilkumar, Manivasagan, Venkatesan, & Kim, 2013; Yang et al., 2008). A considerable reduction in the viability of Lewis lung carcinoma cells and in proliferation rate of human melanoma cells were observed by the treatment of fucoidans isolated from *Sargassum* spp., *Fucus vesiculosus* and *Fucus evanescens* (Ale, Maruyama, Tamauchi, Mikkelsen, & Meyer, 2011; Anastyuk et al., 2012). Fucoidan from *F. vesiculosus* was also reported to be a potent immunomodulator, improving the development and growth of dendritic cells through the induction and release of interleukin (IL)-12 and tumor necrosis factor (TNF)-α (Kim & Joo, 2008). In addition, the *in vivo* immunomodulatory effect of fucoidan was reported by Chen et al. (2012). The biological activities of fucoidans have been correlated with their sulfate content and molecular weight (M_w). According to Cho, Lee, and You (2011), oversulfated fucans exhibited more effective anticancer activity than native fucoidan. Yang et al. (2008) found that the lower M_w fucoidan had stronger growth inhibition on human lung cancer cells, suggesting that structural

Abbreviations: NO, nitric oxide; iNOS, inducible NO synthase; TNF-α, tumor necrosis factor-α; COX-2, cyclooxygenase-2; IL, interleukin; NF-κB, nuclear factor-kappa B; MAPKs, mitogen-activated protein kinases; ERK, including extracellular signal-regulated kinases; JNK, c-Jun N-terminal kinase; p38, mitogen-activated protein kinases.

* Corresponding author. Tel.: +82 33 640 2853; fax: +82 33 640 2340.
E-mail address: umyousg@gwnu.ac.kr (S. You).

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features could be a major factor affecting the biological activity of fucoidans.

Agarum cribrosum is an edible seaweed commonly found in the sub-tidal zone in Northeast Asian countries, including Korea, Japan and China. However, it has been poorly utilized for human consumption and in bio-industry. Various components were characterized in *A. cribrosum*, including carbohydrates, proteins, lipids and polyphenolic compounds, showing different biological activities (Park, Min, & Park, 2012). Among them, phenolic compounds are usually studied in anticancer, antioxidant and neuroprotection activities (Wang et al., 2012; Prasad et al., 2009). It was reported by Cho, Lee, Lee, Lee, and You (2013), the ethanol (EtOH) extracts from *A. cribrosum* exhibited antioxidation, and inhibited nitric oxide (NO) induction from Raw 264.7 cells without cell cytotoxicity. In addition, the ethyl-acetate and butanol sub-fractions derived from the EtOH extracts maintained high levels (80–89%) of the neurons in hippocampal CA1 region at the ischemia-damaged gerbil, suggesting their strong neuroprotective activity (Kim et al., 2014). Besides the work on EtOH extracts of *A. cribrosum*, there are relatively few reports on its aqueous extract containing fucoidans. A basic understanding of the structural characteristics of fucoidans from *A. cribrosum* may lead to a better interpretation of their bioactivities. In this study, the sulfated-fucans from *A. cribrosum* were extracted in a dilute acidic aqueous solution, and subsequently fractionated using ion-exchange chromatography. The objectives of this study were to investigate the chemical and molecular characteristics of the crude and sulfated-fucan fractions, to evaluate their immunomodulatory activity, and to correlate their structures with their bioactivities.

2. Materials and methods

2.1. Materials and reagents

The brown seaweed, *A. cribrosum*, was collected from the coast of Gangneung, Gangwon province, Korea. The seaweed was carefully rinsed with fresh water and dried at 50 °C. The dried raw sample was milled using a blender, sieved (<50 mesh) and stored in plastic bags at −20 °C before extraction of fucoidans. Cell culture media (RPMI 1640), penicillin/streptomycin, fetal bovine serum (FBS) and other materials required for culturing cells were purchased from Lonza Inc. (Walkersville, MD, USA). Griess reagent and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals and other reagents used in this work were of analytical grade.

2.2. Extraction of crude fucoidan

The milled sample (10 g) was treated with 95% EtOH (300 mL) under constant mechanical stirring for 4 h at room temperature (RT). The sample was centrifuged at 3000 × *g* for 10 min, and the sediment was rewashed with EtOH under the same conditions, rinsed with acetone, and then dried at RT in a fume hood. The dried sample (10 g) was extracted with diluted HCl (0.01 N, 500 mL) at 60 °C with stirring for 2 h, centrifuged at 3000 × *g* for 10 min at RT, and the supernatant was collected. The extracted supernatant was concentrated by evaporation under reduced pressure at 60 °C. EtOH (99%) was added to the supernatants to obtain a final concentration of 30%, and the solution was placed at 4 °C for 4 h. After centrifugation at 3000 × *g* for 10 min, more EtOH was added to obtain a final EtOH concentration of 70%, and the solution was kept at 4 °C overnight. The precipitate was isolated by filtration of the solution with a nylon membrane (0.45 μm pore size; Whatman International; Maidstone, UK), washed with EtOH (99%) and acetone, and then dried at RT in a fume hood. The precipitate was re-dissolved in distilled water, dialyzed (MWCO, 8–10 kDa, Millipore) against fresh water for 72 h, and then lyophilized.

2.3. Fractionation of crude fucoidans

The crude fucoidan (200 mg) was dissolved in distilled water (10 mL), and the solution was injected into a DEAE-Sepharose fast flow column (17-0709-01; GE Healthcare Bio-Science AB; Uppsala, Sweden) equilibrated with distilled water. Ten milliliter of fucoidan solution (10 mg/mL) was initially eluted with 800 mL of distilled water to obtain a non-adsorbed fraction, and subsequently eluted with different concentrations of NaCl stepwise increasing from 0.5 to 1.0 M (each 400 mL). Two major fractions, F_1 and F_2, were obtained and determined by the phenol–H_2SO_4 assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) measuring the absorbance at 490 nm. The fractions were extensively dialyzed against distilled water for 3 days and lyophilized.

2.4. Analytical methods

Total carbohydrate content of the fucoidans was estimated by the phenol–H_2SO_4 assay after dissolving the samples (1 mg/mL) in H_2O and using a standard solution with fucose (Dubois et al., 1956). Protein content was measured by the Lowry method using bovine serum albumin (BSA) as a standard (DC Protein Assay kit; Bio-Rad; Hercules, CA, USA) (Lowry, Rosebrough, Farr, & Randall, 1951). Sulfate content of the fucoidans was determined by the $BaCl_2$-gelatin method using K_2SO_4 as a standard after hydrolyzing the fucoidans in 0.5 N HCl at 105 °C for 5 h (Dodgson & Price, 1962). Uronic acid content of the fucoidans was analyzed by a sulfamate/*m*-hydroxydiphenyl colorimetric assay using glucuronic acid as a standard (Filisetti-Cozzi & Carpita, 1991).

2.5. Determination of monosaccharide composition

The monosaccharide composition was carried out according to the method developed by Cao, Lee, and You (2014), using gas chromatography. Briefly, for the monosaccharide composition analysis, the fucoidan was hydrolyzed with 4 M trifluroacetic acid (TFA) at 100 °C for 6 h followed by the reduction of the hydrolysates in water using sodium borodeuteride ($NaBD_4$) and acetylation with acetic anhydride, analyzed by a gas chromatography–mass spectrometry (GC–MS, 6890 N/MSD5973, Agilent Technologies, Santa Clara, CA). The gas chromatograph, equipped with a flame ionization detector using a HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm) was programmed to rise from 180 to 220 °C at 4 °C/min, and then hold at 220 °C for 30 min. The carrier gas was high-purity nitrogen, and the flow rate was 30 mL/min. Sugar standards (L-fucose, D-galactose, D-mannose and D-glucose) were used to identify the sugars based on their retention times.

2.6. Determination of average molecular weight

The weight average M_w of the water-soluble fucoidans was determined by high-performance size exclusion chromatography (HPSEC) linked to multi-angle laser light scattering (MALLS), and a refractive index (RI) detection system. The HPSEC-MALLS-RI system was composed of a Waters 510 pump, Rheodyne model 7072 injector valve with a 200 μL sample loop, a guard column (TSK PW; TosoBiosep; Montgomeryville, PA, USA), a SEC column (TSK G5000 PW, 7.5 × 600 mm; TosoBiosep), a MALLS detector (HELEOS; Wyatt Technology Corp.; Santa Barbara, CA, USA) and an RI detector (model RI-150; Thermo Electron Corp.; Yokohama, Japan). A solution of 0.15 M $NaNO_3$ and 0.02% NaN_3 was used as a mobile phase at a flow rate of 0.4 mL/min. The normalization of the MALLS detector and the determination of volume delay between the MALLS and

RI detectors were carried out using bovine serum albumin (BSA, $dn/dc = 0.185$). The calculations of M_w and radius of gyration (R_g) were carried out using ASTRA version 5.3 software (Wyatt Technology Corp.).

2.7. Carboxyl-reduction of fraction F_1

The reduction of the uronic acids was determined according to a slightly modified method reported by Taylor and Conrad (1972). Briefly, the fraction F_1 (100 mg) was dissolved in distilled water (10 mL), and then reacted (pH 4.7) with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) for 90 min at RT. Sodium borodeuteride ($NaBD_4$, 2.0 M, 10 mL) was added drop-wise, allowed to react for 90 min, and the pH was adjusted to 7.0 using diluted sodium hydroxide (NaOH). The reduced F_1 (RF_1) was dialyzed against distilled water for 3 days and lyophilized.

2.8. Desulfation of carboxyl-reduced fucoidan

The desulfation procedure of RF_1 was conducted according to the method previously reported by Tabarsa, Lee, and You (2012). One hundred milligrams of RF_1 was dissolved in distilled water (10 mL), passed through a cationic exchange column (Dowex 50 W-x8) and lyophilized to yield the fucoidan–pyridinium salts. Desulfation from the fucoidan–pyridinium salts (100 mg) was carried out for 40 min at 120 °C using methanol as a sulfate acceptor. After desulfation, the reaction mixture was dialyzed and lyophilized to obtain the desulfated F_1 (RDF_1).

2.9. Glycosidic linkage analysis

The F_1, RF_1 and RDF_1 were per-methylated according to the method of Ciucanu and Kerek (1984) with slight modifications. The samples (2–3 mg) were dissolved in 0.5 mL of dimethylsulfoxide (DMSO) under nitrogen, and then methylated with 0.3 mL of methyl iodide (CH_3I) and 20 mg of dried NaOH powder. Partially methylated alditol acetate was prepared from the fully methylated sample by acid hydrolysis with 4 M trifluroacetic acid (TFA) at 100 °C for 6 h. The hydrolysates were reduced in water with $NaBD_4$, and acetylated with acetic anhydride. The derivative, a partially methylated alditol acetate, was analyzed by gas chromatography–mass spectrometry (GC–MS; 6890 N/MSD 5973; Agilent Technologies; Santa Clara, CA, USA) in an HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm; Agilent Technologies). Helium was used as the carrier gas at a constant flow rate of 1.2 mL/min. The oven temperature was programmed from 160 to 210 °C in 10 min and 240 °C in 10 min at a speed of 5 °C/min. The inlet temperature was kept constant at 250 °C. The mass range was 35–450 (m/z), and peak assignments were based on retention time and mass spectra.

2.10. NO production

The mouse macrophage cell line (Raw 264.7) was obtained from American Type Culture Collection (ATCC; Manassas, VA, USA), and incubated in RPMI-1640 medium containing 10% FBS. The NO release activity of the fucoidans was determined on the basis of nitrite concentration in macrophage culture supernatants, which was measured by the Griess reaction as described by Green et al. (1982). In brief, Raw 264.7 cells (100 μL of 1×10^6 cells/mL) were plated in a 96-well microplate and incubated for 24 h at 37 °C in the presence of 5% CO_2. The cultured cells were treated with fucoidan solutions (100 μL) at concentrations of 6.25, 12.5 and 25 μg/mL (in triplicates) or lipopolysaccharide (LPS) (1 μg/mL), used as a positive control, and incubated for 24 h at 37 °C. The cultured cell supernatant (100 μL) was mixed with an equal volume of Griess reagent (1%) and incubated at RT for 10 min. The absorbance was measured at 540 nm using an EL-800 microplate reader. The NO production from the Raw 264.7 cells was calculated with reference to a standard curve obtained with $NaNO_2$ (1–200 μM in culture medium).

2.11. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA of Raw 264.7 cells treated with LPS and the fucoidan solutions was extracted using TRIzol reagent (Invitrogen; Carlsbad, CA, USA). The concentration of total RNA was measured by a spectrophotometer before synthesizing cDNA with an oligo$(dT)_{20}$ primer and Superscript III reverse transcriptase (Invitrogen). The resulting cDNA was amplified by PCR using DNA polymerase (GoTaq Flexi, Promega, Madison). The RT-PCR reaction thermocycling program consisted of an initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation (94 °C for 30 s), annealing (56 °C for 40 s), and extension (72 °C for 1 min), and the final extension step at 72 °C for 10 min. The products of RT-PCR were separated by gel electrophoresis using 1% agarose gel stained with ethidium bromide, and the gels were viewed under ultraviolet (UV) transillumination. The sequences of primers used in this experiment were as follows:

(Forward) 5′-CTGCAGCACTTGGATCAGGAACCTG-3′
(Reverse) 5′-GGGAGTAGC CTGTGTGCACCTGGAA-3′ for iNOS;
(Forward) 5′-CCCCCACAGTCAAAGACACT-3′
(Reverse) 5′-GAGTCCATGTTCCAGGAGGA-3′ for COX-2;
(Forward) 5′-TACCTGGTAGAAGTGATGCC-3′
(Reverse) 5′-CATCATGTATGCTTCTATGC-3′ for IL-10;
(Forward) 5′-ATGTGCAAAAAGCTGGCTTTG-3′
(Reverse) 5′-ATTTGTGGTGGATGATGGAGG-3′ for β-actin.

2.12. Western blot analysis

The cellular lysate was centrifuged (12,000 × g for 10 min at 4 °C), and whole cell extract (30 μg protein/lane) was denatured and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated proteins were electro-phoretically transferred from gels to nitrocellulose membranes. Nonspecific binding was blocked with 5% BSA dissolved in Tris-buffered saline with Tween 20 (TBST) at RT for 2 h. The membranes were then washed three times and incubated with the indicated antibodies at 4 °C for overnight. The specific bands were detected with a chemiluminescence ECL kit (Amersham Bio-sciences) and quantified by densitometry using a gel visualizer (Alpha Innotech; San Lenardo, CA).

2.13. Statistical analysis

All experiments were conducted in triplicate ($n = 3$). Data are presented as the mean value with standard deviation. All statistical analyses were performed using SAS (SAS Institute; Cary, NC, USA). Statistical differences were tested by the Student's t-test, one-way analysis of variance (ANOVA), and Duncan's multiple-range test. The critical p values were set at 0.05, and a probability value of $p < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Chemical composition analysis

The yields and proximate compositions of the crude and fractionated fucoidans from *A. cribrosum* are shown in Table 1. The extraction yield of crude fucoidan was 8.1%, which was similar to that of fucoidan isolated from *Undaria pinnatifida* (8.8%) (Yang,

Table 1
Yield and chemical composition of crude and fractionated fucoidans (F_1 and F_2) from *A. cribrosum*.

Component	Sulfated polysaccharides		
	Crude	F_1	F_2
Yield (%)[a]	8.1 ± 1.2	52.0 ± 0.4	20.5 ± 1.9
Total carbohydrate (%)	52.4 ± 2.2	55.7 ± 2.2	56.0 ± 4.8
Sulfate content (%)	18.3 ± 0.9	12.7 ± 0.8	23.0 ± 0.3
Protein (%)	9.3 ± 0.6	4.9 ± 0.3	3.9 ± 0.3
Uronic acid (%)	16.2 ± 2.5	21.8 ± 3.9	14.1 ± 0.4
Monosaccharide content (%)			
Fuc	44.2 ± 0.6	46.7 ± 0.8	44.0 ± 2.0
Xyl	10.7 ± 2.6	17.0 ± 1.4	n.d.[b]
Man	20.4 ± 2.3	26.8 ± 2.0	18.9 ± 1.2
Glc	3.5 ± 0.6	9.5 ± 0.5	n.d.
Gal	16.8 ± 0.6	n.d.	33.9 ± 0.6

[a] Yield—(weight of crude polysaccharide/weight of alga powder) × 100.
[b] n.d.—not detected.

Chung, & You, 2008). However, the yield was significantly higher than that of fucoidans from *Ecklonia cava* and *Laminaria japonica* (1.8 and 2.3%, respectively) (Lee et al., 2012; Wang, Zhang, Zhang, & Li, 2008). The crude fucoidan was mostly composed of carbohydrates (52.4%), uronic acid (16.2%), sulfates (18.3%) and a substantial amount of proteins (9.3%). A similar chemical composition has been observed in the fucoidans from *E. cava* and *Sargassum stenophyllum* (Durate, Cardoso, Noseda, & Cerezo, 2001; Lee et al., 2012). Monosaccharide composition analysis revealed that fucose (44.2%) was the major sugar in the crude fucoidan with considerable amounts of mannose (20.4%), galactose (16.8%) and xylose (10.7%), as well as small amounts of glucose (3.5%). This composition was slightly different from that of *E. cava* as *reported* by Lee et al. (2012), where fucose (61.6%) and galactose (27.2%) were the major sugar units with an absence of mannose.

The crude sulfated fucoidan was fractionated on a DEAE-cellulose anion-exchange chromatography column, which yielded two fractions: F_1 which was mostly non-absorbed polymers eluted with distilled water and F_2 which contained slightly charged polymers eluted with 0.5 M NaCl with yields of 52.0 and 20.5%, respectively. As shown in Table 1, the chemical compositions of F_1 and F_2 contained mainly carbohydrates (55.7 and 56.0%), sulfates (12.7 and 23.0%) and uronic acids (21.8 and 14.1%), with a minor amount of proteins (4.9 and 3.9%). The monosaccharide composition of the F_1 fraction revealed that while fucose (46.7%) and mannose (26.8%) were the major sugar units, considerable amounts of xylose (17.0%) and glucose (9.5%) were also present. On the other hand, fucose (44.0%) and galactose (33.9%) were the major sugar units in fraction F_2, with a substantial amount of mannose (18.9%). However, xylose and glucose were not detected in the F_2 fraction. These results suggested that it was possible to obtain two different fucoidan moieties with various chemical compositions by fractionating crude fucoidan using ion-exchange chromatography, which resulted in the fractionation of crude fucoidan with similar chemical composition and less heterogeneity. In a study of a series of fractionated fucoidans from *S. stenophyllum*, significant variation was observed in the proportion of carbohydrates, sulfates, monosaccharide composition and uronic acid (Durate et al., 2001). This discrepancy in the extraction yield and chemical composition might be due to the differences in the species, in their growth conditions, as well as in the methods used for extraction, purification and analysis (Cho et al., 2013; Lee et al., 2012; Yang et al., 2008a).

3.2. Molecular characteristics of crude and fractionated fucoidans

The overlaid UV and RI chromatograms for the crude and fractionated fucoidans are shown in Fig. 1. As shown in the UV and RI chromatograms, crude fucoidans were eluted from the SEC column with elution times of 24 and 45 min, with two distinct peaks indicating their two different polymer distributions. The average M_w values of the peaks from the crude fucoidan obtained using the MALLS technique were 2420.0×10^3 and 315.9×10^3 g/mol, respectively. Similar M_w values were reported on fucoidan from *U. pinnatifida*, ranging from 500×10^3 to 2400×10^3 g/mol (Yang et al., 2008a), while fucoidan from *E. cava* had significantly lower M_w values ($18–359 \times 10^3$ g/mol), as reported by Lee et al. (2012). These differences in the M_w may be due to differences in the species, the extraction procedure, as well as the analytical methods.

Compared to the crude fucoidan, different elution profiles were observed in the fractions F_1 and F_2 because they had single major peaks at the elution times of 25–45 and 24–40 min, respectively (Fig. 1B and C). The average M_w values of F_1 and F_2 were 110.1×10^3 and 472.3×10^3 g/mol, respectively, showing considerable variation when compared to that of crude fucoidan. A strong UV response was observed in the UV chromatogram of the crude fucoidan, while those of the F_1 and F_2 fractions exhibited relatively weak UV responses, which was in good agreement with the protein content in the crude fucoidan and fractions F_1 and F_2, as shown in Table 1. The radius of gyration (R_g) values, relating to the size of a polymer, of the crude and fractionated fucoidans were 49.0, 51.6 and 43.4 nm, respectively (Table 2). These results indicated that the fractionation of the crude fucoidan by ion-exchange chromatography led to the isolation of sulfated-fucans with considerable variation in their molecular weights and enabled to compare the molecular structure to biological activity.

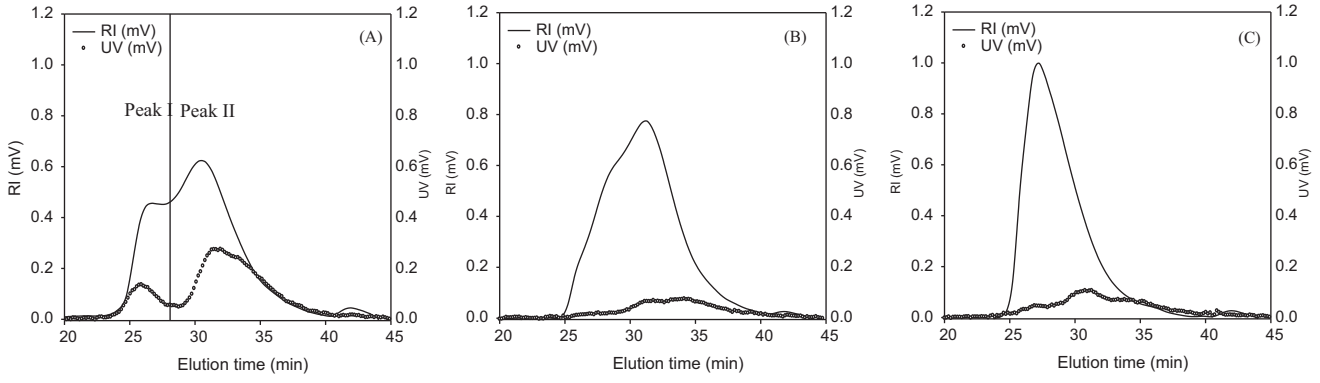

Fig. 1. UV and RI chromatograms of the (A) crude fucoidan, (B) F_1 and (C) F_2 fractions dissolved in distilled water using a TSK G5000 PW column with 0.15 M $NaNO_3$ and 0.02% NaN_3 at a flow rate of 0.4 mL/min.

Table 2
Average molecular weight (M_w) and radius of gyration (R_g) of crude and fractioned fucoidans.

Sample	$M_w \times 10^3$ (g/mol)		R_g (nm)	
	Peak I	Peak II	Peak I	Peak II
Crude	2420.0 ± 0.28	315.9 ± 0.19	38.9 ± 0.14	49.0 ± 1.34
F_1	–	110.1 ± 0.03	–	51.6 ± 2.26
F_2	472.3 ± 0.02	–	43.4 ± 1.76	–

3.3. Immunomodulatory activities of crude and fractioned fucoidans

The murine macrophage cell line (Raw 264.7), releasing immune-related chemokines and cytokines such as NO, prostaglandin E_2 (PGE_2) and interleukins in response to LPS has been used to determine the immunomodulatory activities of compounds by investigating the level of the released chemokines and cytokines (Lee et al., 2012).

The crude fucoidan and fucoidan fractions at different concentrations (6.25, 12.5 and 25.0 μg/mL) did not significantly affect the proliferation of Raw 264.7 cells, suggesting their non-toxic effect on the cells (data not shown). The levels of NO released from Raw 264.7 cells by the crude and fractionated fucoidans at concentrations of 6.25–25.0 μg/mL are shown in Fig. 2A. A considerable amount of NO was released by the crude (17 μM) and F_1 fraction (30 μM) when used at the concentration of 25 μg/mL, despite the fact that these levels of NO production were significantly lower than that of the positive control, LPS (40 μM NO released, 1 μg/mL). On the other hand, a very low level of NO production was observed for fraction F_2, indicating its relatively weak stimulant activity on macrophages when compared to the crude fucoidan and F_1 fraction. Similar trends of nitrite production from peritoneal macrophages were observed with the sulfated polysaccharides from a brown seaweed, *F. vesiculosus*, in which the fucoidans considerably stimulated the cell to produce NO, as well as other cytokines (TNF-α, IL-1 and IL-6) (Choi, Kim, Kim, & Hwang, 2005). We also investigated whether the NO production from Raw 264.7 cells by the crude and fractionated fucoidans was due to the increased mRNA expression of inducible nitric oxide synthetase (iNOS). The induction of iNOS expression was determined by agarose gel analysis of the RT-PCR products with primers for iNOS mRNA.

As shown in Fig. 2B and C, Raw 264.7 cells incubated with crude fucoidan and F_1 fraction exhibited distinct and strong bands in a dose-dependent manner, indicating significantly increased expression of iNOS mRNA, that was considerably higher than that of the F_2 fraction. This suggested that NO release from Raw 264.7 cells could be attributed to the increased expression of iNOS due to the activation of macrophages in the presence of the crude fucoidan and F_1 fraction. A similar trend was observed in the expression of PGE_2 synthetase and cyclooxygenase-2 (COX-2), as determined by gel electrophoresis of RT-PCR products with primers for COX-2. A high level of COX-2 expression from Raw 264.7 cells was observed when treated with crude fucoidan and fraction F_1 (Fig. 3A). Interestingly, the F_2 fraction exhibited a considerable level of COX-2 expression as well. These results implied that the crude fucoidan and both fractions might stimulate Raw 264.7 cells to produce PGE_2. Expression of the anti-inflammatory cytokine, IL-10, was also significantly induced by the crude fucoidan and F_1 fraction (Fig. 3B), which suggested that the crude fucoidan and F_1 fraction might suppress the potential detrimental effect of pro-inflammatory cytokines by releasing IL-10.

Further experiments were carried out to investigate how these fucoidans induced the release of pro-inflammatory and anti-inflammatory mediators in Raw 264.7 cells. Nuclear factor-κB (NF-κB) plays critical roles in inflammation, immunity and cell proliferation. Inactive NF-κB normally binds to inhibitor-κB (I-κB) in the cytosol, and degradation and phosphorylation of I-κB is necessary to release NF-κB from the cytoplasmic NF-κB/I-κB complex to allow its subsequent translocation to the cell nucleus (Nakamura, Suzuki, Wada, Kodama, & Doi, 2006). Fig. 4 shows the expression of I-κB-α, a degraded form of I-κB, upon treatment with the samples, which reveals that the sample molecules induced degradation of I-κB. Therefore, it was likely that the degraded I-κB might lead to the activation of NF-κB, resulting in the stimulation of

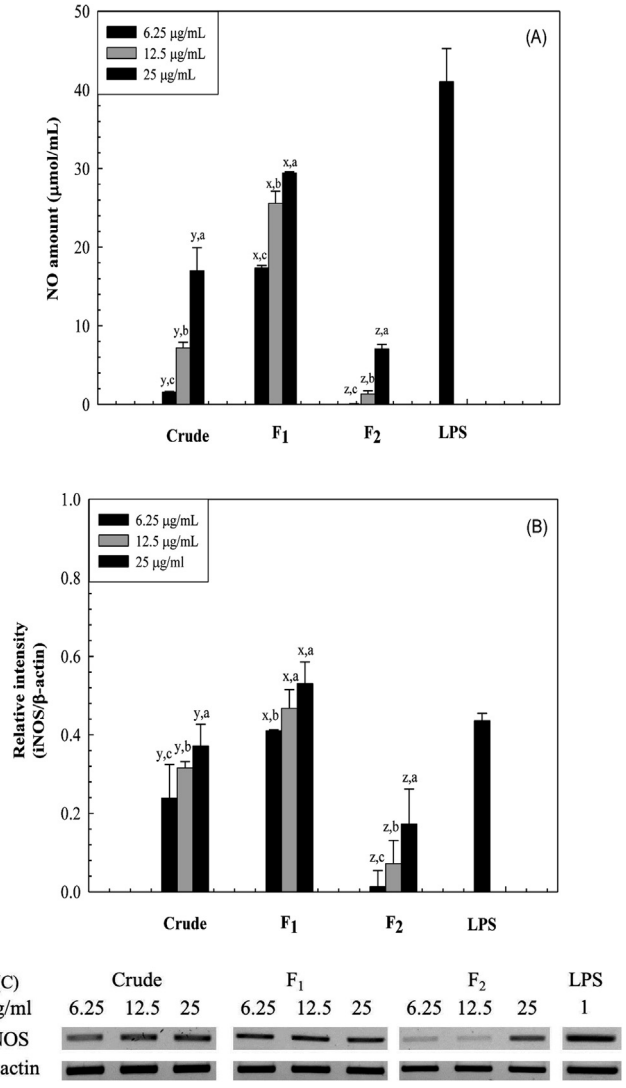

Fig. 2. Amounts of nitric oxide (NO) produced by Raw 264.7 cells following treatment with different concentrations of the crude and fractionated fucoidans. (A) Raw 264.7 cells were incubated for 24 h with different concentrations of fucoidans or 1 μg/mL of LPS (positive control). (B) The iNOS mRNA expression was determined by RT-PCR. (C) Graphic representation of the PCR product for RNA levels of iNOS. The data are presented as the means ± standard deviations ($n = 3$). The letters x, y, z indicate a significant difference ($p < 0.05$) between the concentrations within the crude and fractionated fucoidans, with a, b, c indicating a significant difference ($p < 0.05$) between the crude and fractionated fucoidans at each concentration. β-actin was used as a control.

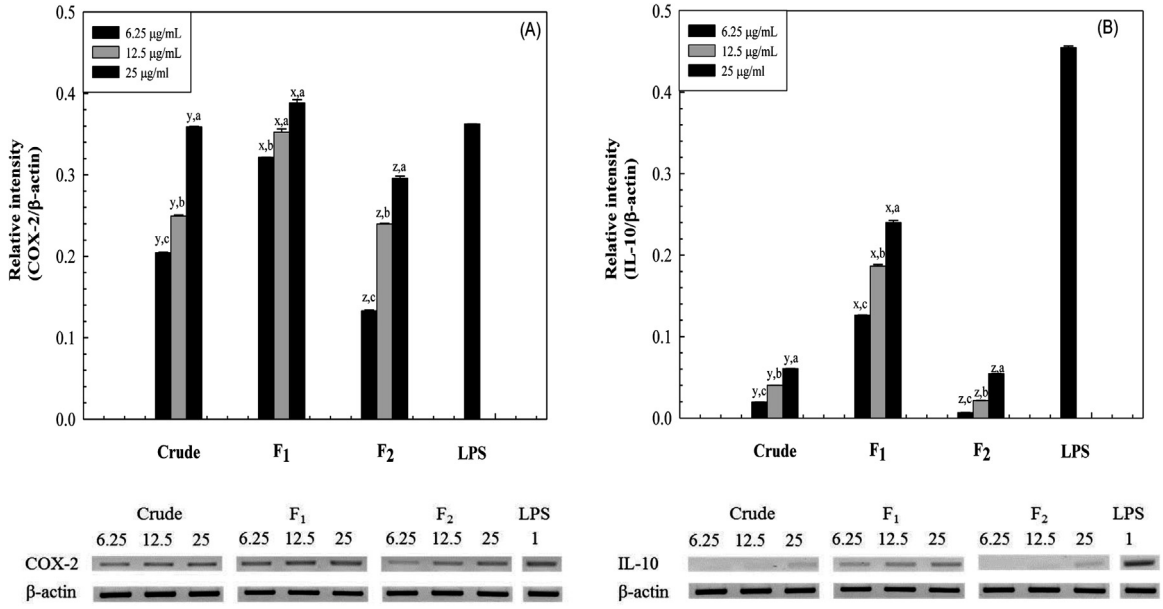

Fig. 3. Effects of fucoidans on COX-2 and IL-10 mRNA expression in Raw 264.7 cells. Raw 264.7 cells were incubated for 24 h with different concentrations of fucoidans or 1 μg/mL of LPS (positive control). (A) Graphic representation of the PCR products for mRNA levels of COX-2 and the cytokine mRNA expressions for COX-2 as determined by RT-PCR. (B) Graph of the PCR products for mRNA levels of IL-10 and the cytokine mRNA expressions for IL-10, as determined by RT-PCR. The data are presented as the means ± standard deviations ($n = 3$). The letters x, y, z indicate a significant difference ($p < 0.05$) between the concentrations within the crude and fractionated fucoidans, with a, b, c indicating a significant difference ($p < 0.05$) between the crude and fractionated fucoidans at each concentration. β-actin was used as a control.

Raw 264.7 cells. Macrophages have been reported to be activated not only by the transcriptional activation of NF-κB but also by phosphorylation of MAPK family members, including extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinase (JNK) and mitogen-activated protein kinases (p38), that play a crucial role in mediating the induction of pro-inflammatory cytokines (Lee et al., 2013). As shown in Fig. 4, the sample treatment significantly induced the phosphorylation of ERK, JNK and p38 because of the stimulation of Raw 264.7 cells. These results, therefore, suggested that the crude and fractionated fucoidans enabled macrophage stimulation via the activation of NF-κB and MAPK pathways.

It was previously reported that the sulfated fucan from *S. fusiforme* induced considerable TNF-α and IL-1β cytokine secretion in murine macrophages, exhibiting strong immunomodulatory activity (Chen et al., 2012). On the other hand, Park et al. (2011) showed strong anti-inflammatory activity of fucoidan, which suppressed NF-κB activation and down-regulated the ERK, JNK, MAPK and AKT pathways in LPS-induced BV2 microglial cells, thereby preventing the detrimental effect of pro-inflammatory cytokines. In a study of other sulfated polysaccharides, Schepetkin et al. (2008) observed a relationship between the M_w and NO production from macrophages, reporting that higher M_w polysaccharides induced a greater amount of NO production. In addition, Leiro, Castro, Arranz, and Lamas (2007) reported that the level of NO production was proportional related to the sulfate content of the polysaccharides. In the current study, such a trend was only weakly shown because F_1 had the lowest amount of sulfate and its M_w was lower than that of F_2 fraction. It was too complex to correlate the structural and biological activities of the sulfated-fucans beyond this preliminary analysis because of their considerable compositional and structural heterogeneity. Further research is necessary to obtain a better understanding of their fine structure and biological activities.

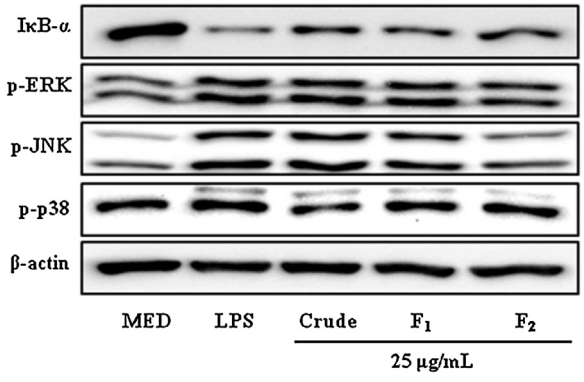

Fig. 4. Effect of fucoidans on I-κB-α and MAPKs phosphorylation in Raw 264.7 cells. Raw 264.7 cells were inoculated in 100-mm dishes, and 12 h after seeding, the cells were treated with 25 μg/mL of fucoidans or 1 μg/mL of LPS (positive control). Whole cell extracts were immunoblotted with indicated antibodies. β-actin was used as a control.

3.4. Glycosidic linkage analysis of F_1 fraction

The nature of the glycosidic linkages and sulfate position of the most immune-enhancing polysaccharides (fraction F_1) was elucidated by methylation analysis of native F_1, carboxyl-reduced F_1 (RF_1), as well as reduced and desulfated F_1 (RDF_1). For this

Table 3
Yield and chemical composition of reduced F_1 (RF_1) and desulfated reduced F_1 (RDF_1) from the F_1 fraction.

Component	RF_1	RDF_1
Yield (%)[a]	64.0 ± 1.8	53.0 ± 2.1
Total carbohydrate (%)	68.9 ± 1.4	55.7 ± 1.3
Sulfate content (%)	9.6 ± 1.2	4.8 ± 1.8
Protein (%)	7.5 ± 0.1	6.0 ± 0.1
Monosaccharide content (%)		
Fuc	27.7 ± 0.3	28.8 ± 1.2
Xyl	8.4 ± 0.1	10.8 ± 0.9
Man	26.0 ± 0.2	24.6 ± 1.4
Glc	32.2 ± 0.3	33.2 ± 1.1
Gal	6.8 ± 0.6	2.6 ± 0.8

[a] Yield—(weight of derivatives/weight of F_1 or RF_1 polysaccharides) × 100.

Table 4
Glycosidic linkage analysis of the constituent sugars of the F_1, RF_1 and RDF_1 fractions from *A. cribrosum*.

Methylation[a]	Retention time (min)	Mass fragmentation (*m*/*z*)	Glycosidic linkage	Peak area (%)		
				F_1	RF_1	RDF_1
2,3,4-Me_3-Fuc	8.49	43,59,72,89,102,118,131,162,175	Fuc-(1→	9.3	8.0	12.9
2,3,4-Me_3-Xyl	8.97	43,59,72,89,102,118,130,162	Xyl-(1→	15.1	11.0	9.6
2,4-Me_2-Fuc	10.53	43,59,72,89,101,118,131,160,174,234	→3)-Fuc-(1→	20.7	16.5	25.2
2,3-Me_2-Fuc	11.85	43,59,72,89,101,113,131,143,160,173,188,203,262	→2,3)-Fuc-(1→	6.6	9.1	3.2
Unmethylated Fuc	12.10	43,59,72,87,100,114,129,144,157,172,189,201,232,290	→2,3,4)-Fuc-(1→	11.4	6.3	0.4
3,4-Me_2-Man	12.40	43,59,71,88,101,129,161,190	→2)-Man-(1→	6.2	3.8	5.7
2,3,6-Me_3-Glc	12.66	43,59,71,87,102,118,129,142,162,173,233	→4)-Glc-(1→	3.8	18.4	19.1
4,6-Me_2-Glc	14.03	43,59,71,87,101,118,129,143,161,202,218,231,262	→2,3)-Glc-(1→	8.6	13.3	13.7
3,6-Me_2-Man	14.37	43,74,88,99,113,130,140,173,190,233	→2,4)-Man-(1→	2.2	1.3	1.6
6-Me-Man	15.29	43,73,87,99,115,129,140,150,190,201,218	→2,4,6)-Man-(1→	16.1	12.3	8.6

[a] 2,4-Me_2-Fuc represented 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-fucitol, etc.

purpose, F_1 samples were permethylated according to Ciucanu and Kerek (1984), followed by hydrolysis, reduction and acetylation. When native F_1 was reduced (RF_1), a significant amount of glucose (32.2%) was observed, indicating that the included glucose in the native F_1 fraction mostly existed in the acidic form (Table 3).

Table 4 shows the type and ratios of glycosidic linkages of F_1, RF_1 and RDF_1, as determined by GC-MS. The major sugar components of native F_1 were (1 → 3)-linked fucose, (1 → 2)-linked mannose and (1 → 4)-linked glucose with non-reducing terminal residues of fucose and xylose, which suggested that the F_1 fraction might consist of a trisaccharide composed of fucose, mannose and glucose. Furthermore, the presence of (1 → 2,3)-linked fucose, (1 → 2,3,4)-linked fucose, (1 → 2,4,6)-linked mannose, (1 → 2,4)-linked mannose and (1 → 2,3)-linked glucose indicated that some branches might exist at the backbone. The RF_1 was found to possess a large proportion of (1 → 3)-linked fucose and (1 → 2,4,6)-linked mannose, as well as a significantly increased proportion of (1 → 4)- and (1 → 2,3)-linked glucose residues compared with those of the fraction $\underline{F}_1$. This might be attributed to the conversion of glucuronic acid into glucose, suggesting that the uronic acids in fraction F_1 likely existed at (1 → 4)- and (1 → 2,3)-linked glucopyranosides. The solvolytic desulfation resulted in elimination of 75% of the sulfates from RF_1 (Table 3). Methylation analysis showed a significant decrease in the (1 → 2,3)- and (1 → 2,3,4)-linked fucose residues when compared with those of F_1 and RF_1, which indicated that the sulfate groups of F_1 fraction were mainly located at the C-2 and C-4 positions in (1 → 2,3)- and (1 → 2,3,4)-linked fucose residues. Results of the current study, therefore, suggest that the strong immunomodulating sulfated fucan, F_1, mainly consisted of (1 → 3)-linked fucose, (1 → 2)-linked mannose and (1 → 4)-linked glucuronic acid, and the sulfates appeared to be located at the C-2 and C-4 positions of (1 → 2,3)- and (1 → 2,3,4)-linked fucose residues.

4. Conclusions

The sulfated-fucans (crude and F_1 fraction) isolated from *A. cribrosum* appeared to considerably stimulate Raw 264.7 cells through the activation of the NF-κB and MAPKs pathways, which resulted in the production of NO and the induction of mRNA expression of various cytokines, implying their potential behavior as immunostimulators and/or biological response modifiers. The maximally immunoenhancing F_1 fraction was mainly composed of (1 → 3)-linked fucose, (1 → 2)-linked mannose and (1 → 4)-linked glucuronic acid with sulfates at the C-2 or C-2 and C-4 positions in (1 → 2,3)- and (1 → 2,3,4)-linked fucose residues. It is still not clear which structural features could play a pivotal role in their biological activity. Further research on their fine structures as well as the binding mechanism between the polysaccharides and cell surface receptors would lead to a better understanding of the relationship between their molecular structures and bioactivities.

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